Title: Low impact of chytridiomycosis on frog recruitment enables persistence in refuges despite high adult mortality

Authors: Ben C. Scheele [a b,†], David A. Hunter [b], Lee F. Skerratt [c], Laura A. Brannelly [c] and Don A. Driscoll [a]

Authors affiliations:

[a] ARC Centre of Excellence for Environmental Decisions, NERP Environmental Decisions Hub, Fenner School of Environment and Society, Australian National University, Canberra, ACT 0200, Australia.

[b] NSW Office of Environment and Heritage, Queanbeyan, NSW 2620, Australia.

[c] One Health Research Group, School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University, Townsville, Queensland 4811, Australia.

† Corresponding author:

Name: Benjamin Scheele

Address: Fenner School of Environment and Society, Forestry Building [48], Australian National University, Canberra ACT 0200, Australia

Phone: +61 4 0045 9104

E-mail: ben.scheele@anu.edu.au

Abstract

The global chytridiomycosis pandemic caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*) is implicated in the apparent extinction or severe decline of over 200 amphibian species. Many declined species now only persist in isolated remnant populations. In this study we examine how remnant populations coexist with *Bd*, focusing on disease impact on adult survival and recruitment potential in the chytridiomycosis-threatened frog *Litoria verreauxii alpina*. Using skeletochronology we found that the adult male population in both 2011 and 2012 was dominated by a two year old age cohort. The lack of recruitment into the three year old cohort in 2012 indicates that annual adult survival is very low. Combined with high *Bd* prevalence and heavy infection burdens, the pathogen appears to drive almost complete mortality of breeding adults over their first breeding season. However, adults successfully mate prior to large increases in disease prevalence that occurs during the breeding season. Infection prevalence among tadpoles and juveniles is low. Exposure to warm water could provide a mechanism for avoiding or clearing *Bd* infection. Relatively low *Bd* prevalence in juveniles prior to dispersal into terrestrial habitat indicates that *Bd* has minimal impact on early life history stages. As such, recruitment is probably high, allowing populations to persist despite low adult survival. This dependence on reliable annual recruitment may explain why remnant populations persist in permanent ponds rather than ephemeral ponds that were historically occupied. New management strategies that focus on increasing recruitment may provide a way forward for the management of disease-threatened amphibian species.

Key words: amphibian decline, *Batrachochytrium dendrobatidis*, chytrid fungus, recruitment, wildlife disease

1. Introduction

Emerging infectious diseases are increasingly threatening wildlife, and have been implicated in recent high profile die-offs in bats, bees and corals (Daszak et al., 2000; Fisher et al., 2012). The amphibian disease, chytridiomycosis, caused by the fungal skin pathogen *Batrachochytrium dendrobatidis* (hereafter *Bd*) has been linked to amphibian declines in Europe, Australia and the Americas (Berger et al., 1998; Lips et al., 2006; Skerratt et al., 2007). Chytridiomycosis has caused the likely extinction of 113 species (Skerratt et al., 2007). Many additional species have experienced declines in abundance and distribution and while some species re-expand (Scheele et al., 2014a), many remain highly restricted in small, remnant populations with endemic *Bd* infection (Briggs et al., 2010; Puschendorf et al., 2011; Phillott et al., 2013). Understanding how remnant populations persist can assist in their conservation and help develop effective response strategies for other chytridiomycosis-threatened species (Scheele et al., 2014b).

Remnant populations might persist through a range of mechanisms including; unfavourable environmental conditions that limit *Bd* growth (Heard et al., 2014), predatory microorganisms that consume infectious zoospores (Schmeller et al., 2013), genetic based resistance (Savage and Zamudio, 2011) and changes in pathogen virulence or evolved host-pathogen interactions (Altizer et al., 2003). Additionally, some species appear to persist despite high *Bd* prevalence and low adult survival (Muths et al., 2011; Phillott et al., 2013). This suggests that compensatory recruitment may be important (Muths et al., 2011; Tobler et al., 2012; Phillott et al., 2013). Consistent with this, simulations indicate that low tadpole infection and subsequent high juvenile frog survivorship can provide a buffer against adult mortality in populations challenged by chytridiomycosis (Louca et al., 2014). However, empirical research investigating the impact of *Bd* on both adult survivorship and recruitment potential in remnant populations remains limited. Given the hypothesized importance of high annual recruitment for buffering populations against *Bd*-induced adult mortality, there is an urgent need to determine the effects of the pathogen on early life history stages and possible mechanisms that may facilitate high recruitment in populations persisting with *Bd*.

Warm environmental conditions may provide a refuge for tadpoles and juveniles to clear *Bd* infection in some species, facilitating high recruitment and buffering populations against *Bd*-induced adult mortality. Temperature is well known to limit *Bd* infection in adults, with exposure to warm conditions in terrestrial (Puschendorf et al., 2011; Rowley and Alford, 2013) and aquatic environments (Forrest and Schlaepfer, 2011; Heard et al., 2014) providing protection against the pathogen. In vitro, *Bd* growth and survival is reduced at temperatures $\geq$ 28 ^{o}C (Piotrowski et al., 2004) and exposure to temperatures of between 27 ^{o}C and 37 ^{o}C has effectively been used to clear infection in a variety of amphibian species in captivity (Chatfield and Richards-Zawacki, 2011; Baitchman and Pessier, 2013). However, whether warm environmental conditions provide protection from *Bd* for larval stages in the wild remains poorly studied despite suggestions such mechanisms could contribute to population persistence (see Doddington et al., 2013).

While maintaining recruitment potential depends on low *Bd* impacts during the vulnerable tadpole-juvenile period, survival through this stage is of little value if individuals subsequently succumb to disease prior to breeding. Thus, it is important to understand the timing of disease impact in adults. Many populations with endemic *Bd* exhibit large seasonal variation in disease frequency, with periods of high prevalence in adults during the breeding season (Kinney et al., 2011) and under favourable climatic conditions (Phillott et al., 2013). For example, *Bd* prevalence can rise dramatically when pond breeding amphibians enter their breeding habitat and are exposed to water-borne zoospores (Fisher et al., 2009; Kinney et al., 2011). The timing of this increase relative to breeding is crucial: if

a substantial proportion of adults are able to breed prior to large increases in disease prevalence and intensity, the pathogen's impact on adult reproductive potential in that season may be limited.

In this study we asked how remnant populations of the endangered frog *Litoria verreauxii alpina* (alpine tree frog) coexist with endemic *Bd* infection. We addressed this question using three lines of evidence: (1) disease impact on adult survival and changes in disease prevalence during the breeding season, (2) survival to the juvenile stage disease-free as a measure of recruitment potential, (3) the role of environmental refugia in protecting tadpoles from infection. *Litoria v. alpina* has experienced major declines attributed to chytridiomycosis (Osborne et al., 1999; Osborne and Hunter, 2003) and is highly susceptible to *Bd* infection (six of 277 adults exposed to *Bd* under laboratory conditions survived, S. Cashins, James Cook University, unpublished results). However, some remnant populations persist and appear to be relatively stable (Osborne et al., 1999); despite high *Bd* prevalence. Given high susceptibility under laboratory conditions, but observed persistence with *Bd* in a small number of locations, we hypothesized that populations maintain sufficient recruitment potential to facilitate persistence despite ongoing *Bd*-induced adult mortality.

By examining both the impact of chytridiomycosis on adult survival and recruitment potential, our study provides important new insights into mechanisms of population coexistence with disease. Our work has implications for the management of species threatened by chytridiomycosis; demonstrating that there is substantial potential for novel strategies to focus on increasing recruitment potential to prevent further extinctions.

2. Materials and methods

2.1. Study area and species

Sampling was replicated across three independent *L. v. alpina* (alpine tree frog) populations (Kiandra, Eucumbene and Three Mile) located in Kosciuszko National Park (35°34'18" N, 148°32'27" W) in south-eastern Australia between August 2011 and March 2013. Population sizes are relatively small and populations likely consist of less than 150 breeding adults (D.H., unpublished results). Populations are separated by distances of between six and 30 km and range in elevation from 1380 to 1475 m. The region has an average winter minimum of -1.1 ^{o}C and a maximum of 3.9 ^{o}C and corresponding summer averages of 9.1 ^{o}C and 19 ^{o}C (BOM, 2014). Rainfall peaks in winter and spring, but is high throughout the year with an annual average of 1700 mm (Cabramurra station 072091).

Litoria v. alpina breed once a year during spring. In late August or early September, adults emerge from torpor and form breeding aggregations, which persist through to late October. Males call from aquatic vegetation and eggs are deposited below the water surface (Anstis, 1976). Eggs hatch after one to two weeks. The tadpole stage is three to four months long and metamorphosis generally begins in December and peaks in January. During the non-breeding season, adults are widely dispersed from breeding habitat and occupy grassland and woodland, while sub-adults occupy terrestrial habitat after dispersal from maternal ponds. *Litoria v. alpina* is the most abundant amphibian species at each site, with two non-declining species, *Crinia signifera* (common eastern froglet) and *Limnodynastes dumerilii* (eastern banjo frog) also present.

136 At each site, *L. v. alpina* breed in several permanent and adjacent temporary waterbodies. Breeding
137 ponds have diameters ranging 3 to 10 m, range in depth from 0.5 to 3.5 m and are highly vegetated.
138 The terrestrial vegetation at the study sites is open sub-alpine grassland dominated by tussock grass
139 species (*Poa fawcettiae* and *P. helmsii*) and sub-alpine woodland dominated by *Eucalyptus pauciflora*
140 and *E. stellulata*. The dominant aquatic and emergent vegetation used by calling males, tadpoles and
141 metamorphs is *Poa costiniana*, *Carex gaudichaudiana*, *Baloskion australe*, and *Myriophyllum*
142 *variifolium*. All ponds occur in natural *L. v. alpina* habitat, but road construction at one site (Three
143 Mile) has impeded stream flow, increasing habitat.

144

145 Amphibian declines had occurred in the study region by the early 1980s, and have been linked with
146 the emergence of *Bd* (Osborne and Hunter, 2003; Hunter et al., 2010). In the sub-alpine zone, *L. v.*
147 *alpina*, *Pseudophryne corroboree* and *P. pengilleyi* have experienced major reductions in abundance
148 and range (Osborne et al., 1999; Hunter et al., 2010). *Litoria v. alpina* is now restricted to a small
149 number of isolated waterbodies scattered throughout its historical distribution (Osborne et al., 1999)
150 and is classified as endangered (Australian Government Department of the Environment, 2014).

151

152 *2.2. Skeletochronology*

153 We collected samples for skeletochronological analysis to determine the age of individuals from each
154 population during the 2011 (Kiandra $n = 36$, Eucumbene $n = 35$ and Three Mile $n = 37$) and 2012
155 (Kiandra $n = 30$, Eucumbene $n = 30$ and Three Mile $n = 30$) breeding seasons. In 2011, sampling
156 occurred between August and October and involved surveys on multiple nights in each population.
157 All individuals encountered in the breeding habitat, including 12 females, four sub-adults and one
158 juvenile were sampled. The relatively low proportion of females sampled compared to males reflects
159 behaviour differences between the sexes. Males have a loud call and often occupy exposed calling
160 positions on emergent vegetation. In contrast, females do not vocalise and are found in less exposed
161 areas and as such, are more difficult to locate. In 2012, only males were sampled and all individuals
162 from a population were sampled on a single night (these samples represent the early season adult
163 sample described below). Adult sex was determined by the presence or absence of male nuptial pads.
164 A single digit from each individual was removed at the base of the third phalange and stored in 70%
165 ethanol. A skin swab was also collected from each individual to test for *Bd* presence (see below).

166

167 Skeletochronology involved decalcifying whole digits in 10% formic acid for 14 h, followed by
168 rinsing in running water for 3 h. Samples were then vertically embedded in paraffin wax and
169 sectioned using a rotary microtome to cut 10 μm sections. The entire third phalange was sectioned to
170 ensure that the mid diaphysis region, which contains the best sections for aging, was identified.
171 Sections were mounted on slides and stained for 30 min using Harris's haematoxylin and mounted
172 with a 60 mm cover-slip using D.P.X. mounting fluid to create a permanent mount. Lines of arrested
173 growth were counted under 400x magnification using a light microscope. Each individual was aged
174 twice without reference to the previous result. When an inconsistent result was obtained, sections
175 were re-examined and if a reliable count could not be obtained, individuals were excluded ($n = 3$ in
176 2011, $n = 4$ in 2012). A small number of samples from 2012 (Kiandra $n = 4$, Eucumbene $n = 1$) were
177 excluded because of difficulties involving staining.

178

179 The accuracy of skeletochronology is dependent on the presence of clearly discernible lines of
180 arrested growth, and is based on the assumption that these lines are consistently deposited annually
181 (Smirina, 1994). Skeletochronology is a reliable method for aging amphibians in regions that
182 experience strong, consistent seasonal variations in climate, such as sub-alpine environments

(Smirina, 1994), and has been validated in the study landscape using repeat sampling of individually identified *P. corroboree* (Hunter, 2000).

2.3. Adult survival

We combined two consecutive years of age-structure data from the three populations to estimate annual adult male survival. We compared the proportion of individuals of age x in a given year with the proportion of individuals in the same cohort in the subsequent year (aged $x + 1$) (Caughley, 1977). The proportion of individuals in each age cohort was very similar and not significantly different between years (Chi-squared test, $P = 0.91$) and thus, the use of proportions was appropriate to account for slight differences in sample size between years. Because the majority of males appear to reach sexual maturity at two years of age, it was not possible to estimate survival rates for the small proportion of individuals that mature at one year old using this method. In addition, we conducted surveys in August, September and October 2012 to recapture individuals from which a toe was removed in 2011. In total, four nights of survey were undertaken in each population in 2012, with each survey involving two or three experienced personnel searching all potential habitat within a site for five to seven hours.

2.4. Batrachochytrium dendrobatidis infection in different life history stages

To investigate the impact of *Bd* on recruitment potential we sampled tadpoles, newly metamorphosed juveniles and adults during the 2012-2013 breeding season. Within a population, each discrete life history stage was sampled on a separate day.

2.4.1. Adults

Adults from each population were sampled for *Bd* infection once at the beginning of the breeding season (August/September) and once at the end of the breeding season (late October) to investigate how the timing of disease transmission related to breeding. Thirty adults from each population were sampled in both time periods ($n = 180$). To ensure a comparable sample, we focused on adult males. The timing of the first sampling relative to frog emergence from torpor at each population varied. At Eucumbene, blocked access roads delayed sampling and the presence of tadpoles indicated breeding activity had commenced several weeks prior to sampling, while at Three Mile and Kiandra sampling was conducted closer to the initial commencement of breeding.

2.4.2. Tadpoles

Tadpoles were sampled in each population on two occasions; first in December when they reached a large enough size to swab, and a second time at the beginning of January prior to metamorphosis. Tadpoles were caught by dip netting and were sampled from multiple locations in each population to ensure a representative sample. Twenty individuals were sampled during each time period ($n = 120$).

2.4.3. Juveniles

Juveniles were sampled on one occasion in each population in late January or early February. We planned two juvenile samplings, however, juveniles dispersed from the breeding habitat following a large rain event and only one sample of 30 individuals from each population was obtained ($n = 90$).

225

226 *2.5. Batrachochytrium dendrobatidis sampling and testing*

227 We used sterile swabs (Medical Wire & Equipment Co. MW 100–100) to sample all life history
228 stages. Adults and juveniles were sampled in a standardised way with three strokes on each side of
229 the abdominal midline, the inner thighs, hands and feet. A new pair of disposable powder-free nitrile
230 gloves was used for each sample. Tadpoles were sampled by lightly brushing the swab tip over
231 mouth parts 10 times. Samples were analysed using real-time quantitative Polymerase Chain
232 Reaction (PCR) following the methodology of Boyle et al. (2004) and Hyatt et al. (2007) with the
233 exception that Qiagen master mix was used instead of Taqman master mix. Samples were analysed in
234 triplicates and were considered positive if all three wells returned a positive reaction (Hyatt et al.,
235 2007).

236

237 *2.6. Tadpole water temperature measurements*

238 We collected the following information from groups of tadpoles surveyed across multiple ponds in
239 each population on three different occasions in December and January separated by approximately
240 eight days: number of tadpoles, depth of tadpoles in the water column, water temperature at the
241 location of tadpoles, total water depth beneath tadpoles and temperature at maximum depth. All
242 measurements were collected between 12:00 and 15:30. Groups of tadpoles were chosen by selecting
243 a random location in each pond and then measuring the closest group of tadpoles. Locations were
244 selected by measuring the length and width of a pond and then using a random numbers table to
245 define a position within the pond. Locations were constrained for logistical reasons to within two
246 metres of the pond edge. In total, measurements were recorded for 103 groups of tadpoles. We also
247 tested water pH and salinity (measured by electrical conductivity) during adult, tadpole and juvenile
248 sampling. Both pH (Piotrowski et al., 2004) and water salinity (Heard et al., 2014) can limit *Bd*
249 growth, however, no values indicative of conditions unfavourable to *Bd* were recorded (pH range 6 –
250 6.7, electrical conductivity range 17.9 – 51.3 $\mu S\ cm^{-1}$).

251

252 *2.7. Statistical analysis*

253 We used generalised linear models (GLM) to analyse the 2012-2013 *Bd* infection data. First, we
254 investigated *Bd* prevalence in different life history stages using a binomial GLM with *Bd*
255 presence/absence as the response variable and life history stage (adult, juvenile, tadpole), population
256 and an interaction between the two as potential explanatory variables. Next, we investigated *Bd*
257 prevalence and infection intensity in early and late season adult samples using a binomial GLM for
258 prevalence data and a negative binomial GLM for infection intensity data (using the R package
259 MASS, Ripley et al., 2013). For both models, sampling period, population and an interaction between
260 the two were potential explanatory variables. Finally, we used a binomial GLM to investigate *Bd*
261 infection prevalence in juveniles with *Bd* presence/absence as the response variable and population as
262 an explanatory variable. We used Chi-square tests to assess the significance of explanatory variables
263 in all models (Zuur et al., 2007). We did not compare infection intensities between life history stages
264 because differences in infection intensity could result from life stage size differences. Because of
265 very low *Bd* prevalence, tadpole infection data was not analysed separately. All statistical analyses
266 were implemented in the R environment (R Development Core Team, 2014).

267

268 **3. Results**

269 *3.1. Adult survival*

Based on the population age structure data, the annual adult male survival rate was very low (0.04). In both 2011 and 2012, breeding aggregations were dominated by a two year old adult male cohort. This result was consistent across all three populations. In 2011, 10 of 88 sexually mature males were one year old, 77 two years old and only one was three years old. In 2012, 78 of 81 sexually mature males were two years old and three were three years old. The small number of three year old males in 2012 indicates that very few two year old males survived following the 2011 breeding season to return as three year olds in 2012. Consistent with the low rate of survival, no individuals marked in 2011 were recaptured in 2012, despite 12 nights of survey throughout the 2012 breeding season. In 2011, three of 12 females were two years old and nine were three years old. The four sub-adults were one year old and one juvenile displayed no lines of arrested growth. Seventy three of 108 individuals sampled in 2011 were infected with *Bd* (see appendix A for detailed information on population prevalence and infection intensities).

3.2. Batrachochytrium dendrobatidis infection in different life history stages

Batrachochytrium dendrobatidis was detected in all *L. v. alpina* life history stages in 2012-2013. Probability of infection was significantly higher in adults compared to juveniles and tadpoles (P (Chi) = <0.0001, df = 2, 387) (Fig. 1a). Probability of infection was also significantly different among populations (P (Chi) = <0.0001, df =2, 385) (Fig. 1b).

3.2.1. Adults

A total of 180 adults were sampled for *Bd* infection and overall prevalence across time periods and populations was 68%. Probability of infection increased significantly during the breeding season (P (Chi) = 0.0004, df = 1, 178) and was significantly different among the three populations (P (Chi) = 0.0004, df = 2, 176), with a significant interaction between sampling period and population (P (Chi) = 0.03, df = 2, 174) (Fig. 2a). Infection intensity also increased significantly through the breeding season (P (Chi) = 0.01, df = 1, 117) (Fig. 2b). During the late season sampling, two individuals exhibited symptoms of severe chytridiomycosis (most noticeably, loss of righting reflex, Voyles et al., 2009) and one dead individual was observed.

3.2.2. Juveniles

Ninety juveniles were sampled for *Bd* and overall infection prevalence was 17%. Probability of infection was significantly different among the three populations (P (Chi) = 0.03, df = 1, 58) (Fig. 3a). For all infected juveniles, mean infection intensity was 1945 (*S.E.* = 876) zoospore equivalents, with infected juveniles exhibiting a range of infection intensities (Fig. 3b).

Approximately one month after the first juvenile sampling we attempted a second sampling for each population. Despite comprehensive nocturnal surveys and day time vegetation searches, only a very small number of juveniles (n = 6) were detected across the three populations. A large rain event followed the first round of juvenile sampling and it is likely that juveniles dispersed into surrounding terrestrial habitat at this time.

3.2.3. Tadpoles

Infection prevalence in the 120 tadpoles sampled was very low, with only 2.5% (*C.I.* = 0.6 - 8%) of individuals positive. Infection intensities for the three infected tadpoles were 166, 13, 3 zoospore equivalents, respectively.

3.3. Water temperatures

Water temperatures were recorded on three separate occasions in each population approximately eight days apart during late December and January. Measurements were recorded for a total of 103 groups of tadpoles. Tadpoles were observed in water temperatures ranging from 24 ºC to 35 ºC (Fig. 4). Sample days had daily maximum air temperatures ranging from 22 ºC to 30 ºC. The average daily maximum air temperature at Cabramurra (automatic weather station) for December 2012 and January 2013 was 18 ºC and 23 ºC, respectively. During sampling, metamorphosing individuals were observed basking in direct sunlight on emergent aquatic vegetation.

4. Discussion

Determining how remnant populations of chytridiomycosis-threatened amphibians coexist with *Bd* may prevent further extinctions. By focusing on both the impact of *Bd* on adult survival and recruitment potential, our study provides crucial new insights into how populations persist with endemic *Bd*. We show that adult survivorship between the 2011 and 2012 breeding seasons was very low, likely related to high levels of *Bd*-induced mortality. However, despite nearly all adults dying during their first breeding season, remnant populations are relatively stable. Recruitment must therefore be offsetting low adult survival to allow population persistence. We suggest high recruitment potential is maintained by low pathogen impact in early life history stages combined with successful mating by first time breeders. Importantly, first time breeding occurs prior to large increases in *Bd* prevalence and infection intensity that develop as the breeding season progresses. Our results highlight the potential for new management strategies to increase recruitment capacity in chytridiomycosis-threatened populations to reduce risk of extinction.

We found that breeding aggregations of *L. v. alpina* are dominated by a two year old cohort, indicating that most males reach sexual maturity in two years, while a small proportion take one year. In contrast, skeletochronological analysis of museum *L. v. alpina* specimens collected pre-*Bd* emergence, demonstrates populations had multiple adult age cohorts, including males up to six years old (B. S., unpublished results). The dominance of a single two year old age cohort in both years indicates that the populations experience *Bd*-induced die-offs during the breeding season, similar to those described following *Bd* emergence in naïve populations (e.g. Lips et al., 2006; Vredenburg et al., 2010). This conclusion is supported by; 1) the very low rate of recruitment into the three year old cohort in 2012 and the absence of recaptures between breeding seasons, 2) large increases in infection prevalence and intensity during the breeding season (Fig. 2a), 3) observations of dead and moribund individuals, and 4) high species susceptibility under laboratory conditions (S. Cashins, James Cook University, unpublished results). Females also appear to have low survivorship, with most individuals reaching sexual maturity in three years (likely related to larger body size), and a small proportion taking two years. Previous studies have documented reduced adult survivorship associated with *Bd* infection in populations of other declined species (Muths et al., 2011; Phillott et al., 2013), however, the level of infection prevalence and rate of turnover of individuals in the populations we describe appears to be substantially higher.

The low rates of adult survival imply that population persistence is facilitated by high levels of recruitment. In other species, tadpoles can commonly be infected with *Bd*, but mortality is rare until metamorphosis, when infected individuals may succumb to chytridiomycosis (Fisher et al., 2009).

However, we found that *Bd* prevalence remained very low in tadpoles (Fig. 1a), leading to low prevalence in juveniles prior to dispersal from maternal ponds (Fig. 1a). High infection burdens were observed in 8 % of juveniles suggesting that *Bd* causes a small amount of early life history mortality (Fig. 3b). Following dispersal, juveniles occupy terrestrial habitat where the risk of contracting *Bd* is likely low (e.g. Kinney et al., 2011; Hossack et al., 2013), and it is plausible that many remain *Bd* negative during their sub-adult phase. This is congruent with the relatively low prevalence of *Bd* in adults early in the season (Fig. 2a), likely driven by an influx of first time breeders into the adult population. Importantly, first time breeders are able to successfully mate prior to significant increases in disease prevalence and intensity that occur later in the season. This increase is likely related to breeding behaviour, with individuals congregating to breed under conditions that promote continual reinfection from water borne zoospores and favourable temperatures for *Bd* growth (c.f. Briggs et al., 2010; Kinney et al., 2011).

Remnant *L. v. alpina* populations are associated with relatively permanent waterbodies (Osborne et al., 1999). In contrast, the majority of historical records for this species are from ephemeral wetlands (Osborne et al., 1999). The persistence of remnant populations at sites with long hydroperiods is likely related to the species' dependency on consistent annual recruitment when challenged by *Bd*. Ephemeral sites periodically experience drought-induced recruitment failure and under such conditions tadpoles are unable to buffer against disease-induced adult mortality. Given this dependence on annual recruitment, we suggest that remnant populations of *L. v. alpina* persisting with *Bd* are highly vulnerable to stochastic events, where recruitment failure in any one year is likely to cause population extinction.

Frequent exposure to water temperatures exceeding 28 ºC likely explains the low prevalence of *Bd* in *L. v. alpina* tadpoles (Fig. 4). In contrast, adult *L. v. alpina* are not exposed to temperatures that exceed the thermal maxima of *Bd* during the breeding season (water and air temperatures < 20 ºC, B. S., unpublished results). In vitro, *Bd* growth and survival is greatly reduced at temperatures ≥ 28 ºC (Piotrowski et al., 2004) and in the wild, high water temperatures have been linked to low *Bd* prevalence in adults of several species (Forrest and Schlaepfer, 2011; Heard et al., 2014). Under laboratory conditions most *Alytes obstetricans* tadpoles exposed to water temperatures between 26 ºC and 30 ºC cleared *Bd* infection (Geiger et al., 2011) and in the same species, Böll et al. (2012) have suggested that tadpole exposure to warm water in small, shallow ponds may be associated with low *Bd* prevalence. Daskin et al. (2011) demonstrated that one hour daily exposure to 33 ºC significantly reduced *Bd* growth rates over four days, indicating that short-term exposure to such temperatures can limit *Bd*. Because tadpoles of many pond breeding species preferentially occupy warm microhabitats to increase development rates (Wilbur, 1980), we suggest that exposure to warm water temperatures may be an important factor limiting *Bd* infection in tadpoles of other species and warrants further investigation.

While regular exposure to warm water provides a plausible explanation for the low prevalence of *Bd* in *L. v. alpina* tadpoles, we cannot rule out alternative explanations. Because *L. v. alpina* tadpoles feed in the water column, low prevalence could be related to limited tadpole exposure to *Bd* zoospores, despite overlap between adult and tadpole life history stages. In other *Litoria* species, infection prevalence in tadpoles is related to tadpole feeding behaviour, with lower prevalence in water column feeders compared to bottom feeders that scrape algae from rock surfaces (Skerratt et al., 2010). Another potential explanation is that *L. v. alpina* tadpoles have inherently low susceptibility to *Bd* infection, however, we note that *L. v. alpina* tadpoles have substantial keratinized mouth parts and at least one tadpole had a moderately intense infection.

In addition to differences in *Bd* prevalence between life history stages, we document significant differences in *Bd* prevalence between populations (Fig. 1b). Juvenile infection prevalence was highest in the Eucumbene population (Fig. 3a). This site supports a substantial population of *C. signifera*, a non-declining species that is commonly infected with heavy *Bd* loads (B. S., unpublished results). *Crinia signifera* may be a reservoir host for *Bd*, amplifying prevalence in *L. v. alpina* juveniles prior to their dispersal from wetland habitat, however this requires further research. The Eucumbene population was also the only population not to show a significant difference in *Bd* prevalence between adults sampled early and late in the breeding season. This may be explained by the relatively longer period of frog activity between emergence from torpor and the first sampling at this population.

The management of chytridiomycosis-threatened species is a major conservation challenge, with little applied research to guide amphibian disease management (Woodhams et al., 2011; Scheele et al., 2014b). Our results highlight a new avenue for the management of chytridiomycosis. Previous approaches have largely focused on reducing disease impact in adults (reviewed in Woodhams et al., 2011), and while this approach is logical given severe disease impact in this life history stage, our results indicate that alternative strategies focused on increasing recruitment potential or protecting populations from recruitment failure (e.g. drought in the study region Scheele et al., 2012) could be successful. For example, in pond breeding species this may involve the creation of breeding habitat with shallow areas that warm up rapidly to allow tadpoles to thermoregulate in warm water to avoid *Bd* infection. Crucially, ponds must have sufficient hydroperiod to prevent desiccation prior to metamorphosis in dry years. Sufficient pond hydroperiod can be maintained through deepening or lining ponds and water supplementation (Shoo et al., 2011). Similarly, water temperatures can be increased through canopy modification and the use of dark pond linings (Scheele et al., 2014b). Artificial habitat may be created at locations where focal species have been extirpated, but *Bd* remains an ongoing barrier to population re-establishment or in areas with low suitability for *Bd*. Such approaches could be combined with existing strategies that directly target a reduction in *Bd* prevalence (see Woodhams et al., 2011; Scheele et al., 2014b) to minimise the threat posed by chytridiomycosis.

5. Conclusions

Batrachochytrium dendrobatidis has devastated amphibians globally (Skerratt et al., 2007; Fisher et al., 2009) and is now endemic in remnant populations of many declining species (Briggs et al., 2010; Hunter et al., 2010; Puschendorf et al., 2011). Understanding how remnant populations persist with chytridiomycosis is central to their conservation and for guiding the restoration of other species that are largely extirpated from the wild but are retained in captivity (Scheele et al., 2014b). Our study showed that remnant populations challenged by endemic *Bd* can persist despite low annual survival in adults because the pathogen does not severely compromise recruitment potential. Low *Bd* prevalence in early life history stages is likely mediated by high water temperatures providing refuge from *Bd*. We suggest that new management strategies that focus on increasing recruitment capacity could contribute to minimising the risk of extinction in chytridiomycosis-threatened species.

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457 **References**

458 Altizer, S., Harvell, D., Friedle, E., 2003. Rapid evolutionary dynamics and disease threats to
459 biodiversity. Trends Ecol. Evol. 18, 589-596.
460 Anstis, M., 1976. Breeding biology and larval development of *Litoria verreauxii* (Anura: Hylidae).
461 Trans. R. Soc. S. Aust. 100, 193–202.
462 Australian Government Department of the Environment, 2014. *Litoria verreauxii alpina* - Alpine Tree
463 Frog, Verreaux's Alpine Tree Frog. Department of the Environment, Canberra.
464 http://www.environment.gov.au/cgi-bin/sprat/public/publicspecies.pl?taxon_id=66669 (accessed
465 February 2014).
466 Baitchman, E.J., Pessier, A.P., 2013. Pathogenesis, diagnosis, and treatment of amphibian
467 chytridiomycosis. Vet Clin North Am Exot Anim Pract 16, 669-685.
468 Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R.,
469 Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G., Parkes, H., 1998.
470 Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests
471 of Australia and Central America. Proc. Natl. Acad. Sci. USA 95, 9031-9036.
472 Böll, S., Tobler, U., Geiger, C.C., Hansbauer, G., Schmidt, B.R., 2012. The amphibian chytrid fungus
473 in Bavarian populations of *Alytes obstetricans*: past absence, current presence, and metamorph
474 mortality. Amphib-reptil. 33, 319-326.
475 BOM, 2014. (Bureau of Meteorology) Climate statistics for Australian locations. Australian Bureau of
476 Meteorology, Melbourne. http://www.bom.gov.au/climate/data/ (accessed December 2013).
477 Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T., Hyatt, A.D., 2004. Rapid quantitative detection
478 of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman
479 PCR assay. Dis. Aquat. Org. 60, 141-148.
480 Briggs, C.J., Knapp, R.A., Vredenburg, V.T., 2010. Enzootic and epizootic dynamics of the chytrid
481 fungal pathogen of amphibians. Proc. Natl. Acad. Sci. USA 107, 9695-9700.
482 Caughley, G., 1977. Analysis of vertebrate populations. Wiley, New York.
483 Chatfield, M., Richards-Zawacki, C., 2011. Elevated temperature as a treatment for *Batrachochytrium*
484 *dendrobatidis* infection in captive frogs. Dis. Aquat. Org. 94, 235-238.
485 Daskin, J.H., Alford, R.A., Puschendorf, R., 2011. Short-term exposure to warm microhabitats could
486 explain amphibian persistence with *Batrachochytrium dendrobatidis*. PloS ONE 6, e26215.
487 Daszak, P., Cunningham, A.A., Hyatt, A.D., 2000. Wildlife ecology - Emerging infectious diseases of
488 wildlife - Threats to biodiversity and human health. Science 287, 443-449.
489 Doddington, B.J., Bosch, J., Oliver, J.A., Grassly, N.C., Garcia, G., Schmidt, B.R., Garner, T.W.J.,
490 Fisher, M.C., 2013. Context-dependent amphibian host population response to an invading pathogen.
491 Ecology 94, 1795-1804.
492 Fisher, M.C., Garner, T.W.J., Walker, S.F., 2009. Global emergence of *Batrachochytrium*
493 *dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annu. Rev. Microbiol. 63,
494 291-310.
495 Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L., Gurr, S.J.,
496 2012. Emerging fungal threats to animal, plant and ecosystem health. Nature 484, 186-194.
497 Forrest, M.J., Schlaepfer, M.A., 2011. Nothing a hot bath won't cure: Infection rates of amphibian
498 chytrid fungus correlate negatively with water temperature under natural field settings. PLoS ONE 6,
499 e28444.
500 Geiger, C.C., Kupfer, E., Schar, S., Wolf, S., Schmidt, B.R., 2011. Elevated temperature clears
501 chytrid fungus infections from tadpoles of the midwife toad *Alytes obstetricans*. Amphib-reptil. 32,
502 276-280.
503 Heard, G.W., Scroggie, M.P., Clemann, N., Ramsey, D.S.L., 2014. Wetland characteristics influence
504 disease risk for a threatened amphibian. Ecol. Appl. 24, 650–662.
505 Hossack, B.R., Lowe, W.H., Ware, J.L., Corn, P.S., 2013. Disease in a dynamic landscape: host
506 behavior and wildfire reduce amphibian chytrid infection. Biol. Conserv. 157, 293-299.
507 Hunter, D., 2000. The conservation and demography of the southern corroboree frog (*Pseudophryne*
508 *corroboree*). Masters thesis. University of Canberra, Canberra, Australia. p. 138.

509 Hunter, D.A., Speare, R., Marantelli, G., Mendez, D., Pietsch, R., Osborne, W., 2010. Presence of the
510 amphibian chytrid fungus *Batrachochytrium dendrobatidis* in threatened corroboree frog populations
511 in the Australian Alps. Dis. Aquat. Org. 92, 209-216.
512 Hyatt, A.D., Boyle, D.G., Olsen, V., Boyle, D.B., Berger, L., Obendorf, D., Dalton, A., Kriger, K.,
513 Hero, M., Hines, H., Phillott, R., Campbell, R., Marantelli, G., Gleason, F., Colling, A., 2007.
514 Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Dis.
515 Aquat. Org. 73, 175-192.
516 Kinney, V.C., Heemeyer, J.L., Pessier, A.P., Lannoo, M.J., 2011. Seasonal pattern of
517 *Batrachochytrium dendrobatidis* infection and mortality in *Lithobates areolatus*: affirmation of
518 Vredenburg's "10,000 Zoospore Rule". PLoS ONE 6, e16708.
519 Lips, K.R., Brem, F., Brenes, R., Reeve, J.D., Alford, R.A., Voyles, J., Carey, C., Livo, L., Pessier,
520 A.P., Collins, J.P., 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical
521 amphibian community. Proc. Natl. Acad. Sci. USA 103, 3165-3170.
522 Louca, S., Lampo, M., Doebeli, M., 2014. Assessing host extinction risk following exposure to
523 *Batrachochytrium dendrobatidis*. Proc. R. Soc. Lond. B Biol. Sci. 281, 20132783.
524 Muths, E., Scherer, R.D., Pilliod, D.S., 2011. Compensatory effects of recruitment and survival when
525 amphibian populations are perturbed by disease. J. Appl. Ecol. 48, 873-879.
526 Osborne, W., Hunter, D., 2003. Frog declines inthe Snowy Mountains: what do we know after fifteen
527 years? Proceedings of an International Year of Mountains Conference. Australian Alps Liaison
528 Committee.
529 Osborne, W., Hunter, D., Hollis, G., 1999. Population declines and range contraction in Australian
530 alpine frogs, In Declines and disappearances of Australian frogs. ed. A. Campbell, pp. 145-157.
531 Environment Australia, Canberra.
532 Phillott, A.D., Grogan, L.F., Cashins, S.D., McDonald, K.R., Berger, L., Skerratt, L.F., 2013.
533 Chytridiomycosis and seasonal mortality of tropical stream-associated frogs 15 years after
534 introduction of *Batrachochytrium dendrobatidis*. Conserv. Biol. 27, 1058-1068.
535 Piotrowski, J.S., Annis, S.L., Longcore, J.E., 2004. Physiology of *Batrachochytrium dendrobatidis*, a
536 chytrid pathogen of amphibians. Mycologia 96, 9-15.
537 Puschendorf, R., Hoskin, C.J., Cashins, S.D., McDonald, K., Skerratt, L.F., VanDerWal, J., Alford,
538 R.A., 2011. Environmental refuge from disease-driven amphibian extinction. Conserv. Biol. 25, 956-
539 964.
540 R Development Core Team, 2014. *R: A language and environment for statistical computing*. Vienna:
541 R Foundation for Statistical Computing. ISBN 3-900051-07-0, http://www.R-project.org.
542 Ripley, B., Venables, B., Bates, D.M., Hornik, K., 2013. MASS. R package, version 7.3-33
543 http://cran.r-project.org/web/packages/MASS/index.html. (accessed December 2013).
544 Rowley, J.J.L., Alford, R.A., 2013. Hot bodies protect amphibians against chytrid infection in nature.
545 Sci Rep 3, e1515.
546 Savage, A.E., Zamudio, K.R., 2011. MHC genotypes associate with resistance to a frog-killing
547 fungus. Proc. Natl. Acad. Sci. USA 108, 16705-16710.
548 Scheele, B., Driscoll, D., Fischer, J., Hunter, D., 2012. Decline of an endangered amphibian during an
549 extreme climatic event. Ecosphere 3, e101.
550 Scheele, B.C., Guarino, F., Osborne, W., Hunter, D.A., Skerratt, L.F., Driscoll, D.A., 2014a. Decline
551 and re-expansion of an amphibian with high prevalence of chytrid fungus. Biol. Conserv. 170, 86-91.
552 Scheele, B.C., Hunter, D.A., Grogan, L., Berger, L., Kolby, J., McFadden, M., Marantelli, G.,
553 Skerratt, L.F., Driscoll, D.A., 2014b. Interventions for reducing extinction risk in chytridiomycosis-
554 threatened amphibians. Conserv. Biol. 28, 1195–1205.
555 Schmeller, D.S., Blooi, M., Martel, A., Garner, T.W., Fisher, M.C., Azemar, F., Clare, F.C., Leclerc,
556 C., Jäger, L., Guevara-Nieto, M., 2013. Microscopic aquatic predators strongly affect infection
557 dynamics of a globally emerged pathogen. Curr. Biol. 24, 176-180.
558 Shoo, L.P., Olson, D.H., McMenamin, S.K., Murray, K.A., Van Sluys, M., Donnelly, M.A., Stratford,
559 D., Terhivuo, J., Merino-Viteri, A., Herbert, S.M., Bishop, P.J., Corn, P.S., Dovey, L., Griffiths, R.A.,
560 Lowe, K., Mahony, M., McCallum, H., Shuker, J.D., Simpkins, C., Skerratt, L.F., Williams, S.E.,
561 Hero, J.M., 2011. Engineering a future for amphibians under climate change. J. Appl. Ecol. 48, 487-
562 492.

563 Skerratt, L.F., Berger, L., Speare, R., Cashins, S., McDonald, K.R., Phillott, A.D., Hines, H.B.,
564 Kenyon, N., 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of
565 frogs. EcoHealth 4, 125-134.
566 Skerratt, L.F., McDonald, K.R., Hines, H.B., Berger, L., Mendez, D., Phillott, A.D., Cashins, S.D.,
567 Murray, K.A., Speare, R., 2010. Application of the survey protocol for chytridiomycosis to
568 Queensland, Australia. Dis. Aquat. Org. 92, 117-129.
569 Smirina, E., 1994. Age determination and longevity in amphibians. Gerontology 40, 133-146.
570 Tobler, U., Borgula, A., Schmidt, B.R., 2012. Populations of a susceptible amphibian species can
571 grow despite the presence of a pathogenic chytrid fungus. PLoS ONE 7, e34667.
572 Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W.F., Dinudom, A., Cook, D., Webb, R.,
573 Alford, R.A., Skerratt, L.F., Speare, R., 2009. Pathogenesis of chytridiomycosis, a cause of
574 catastrophic amphibian declines. Science 326, 582-585.
575 Vredenburg, V.T., Knapp, R.A., Tunstall, T.S., Briggs, C.J., 2010. Dynamics of an emerging disease
576 drive large-scale amphibian population extinctions. Proc. Natl. Acad. Sci. USA 107, 9689-9694.
577 Wilbur, H.M., 1980. Complex life cycles. Annu. Rev. Ecol. Syst. 11, 67-93.
578 Woodhams, D.C., Bosch, J., Briggs, C.J., Cashins, S., Davis, L.R., Lauer, A., Muths, E., Puschendorf,
579 R., Schmidt, B.R., Sheafor, B., Voyles, J., 2011. Mitigating amphibian disease: strategies to maintain
580 wild populations and control chytridiomycosis. Frontiers in Zoology 8, DOI: 10.1186/1742-9994-
581 1188-1188.
582 Zuur, A.F., Ieno, E.N., Smith, G.M., 2007. *Analysing ecological data*. Springer, New York.

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586 Figure 1. Predicted probability of *Bd* infection during the 2012-2013 breeding season in (a) three *L. v.*
587 *alpina* life history stages, and (b) different *L. v. alpina* populations (overall adult infection shown).
588 Error bars indicate standard error.

589

590 Figure 2. (a) Predicted probability of infection, and (b) predicted infection intensity in adult *L. v.*
591 *alpina* through the 2012-2013 breeding season. Error bars indicate standard error. Early season
592 samples were collected between late August and early September, while late season samples were
593 collected in late October.

594

595 Figure 3. (a) Predicted probability of *Bd* infection in juvenile *L. v. alpina* in different populations (no
596 infection detected at Three Mile). Error bars indicate standard error. (b) Proportion of infected
597 juveniles in four infection intensity categories (total number of infected juveniles = 15/90).
598

599 Figure 4. Water temperature measurements for 103 groups of tadpoles across the three populations.
600 The bold line is the median and boxes represent the 25^{th} and 75^{th} percentiles and error bars show the
601 minimum and maximum values.

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